STUDY ON BIOETHANOL PRODUCTION FROM OIL PALM TRUNK (OPT) SAP BY USING *SACCHAROMYCES CEREVISIAE* KYOKAI NO.7 (ATCC 26422)

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ABSTRACT

Bioethanol is by far the most important biofuel worldwide. This is mainly a result of the proven and established process technology in addition to the wide range of raw materials suitable for its production. The aim of this study is the screening of parameter of pH (3-7) and agitation speed (110-250 rpm) that are significance to the fermentation process for the production of bioethanol from oil palm trunk (OPT) sap by yeast *Saccharomyces cerevisiae* Kyokai no.7(ATCC 26422). Fermentation design was set up to gain fermentation profile and validation to further obtaining the data for the screening of parameter for the whole fermentations process at several points of times. Data of ethanol, glucose and cell dry weight concentration has been collected and analyzed. ANOVA was performed to determine interacting factor affecting bio-ethanol production after the main effect was estimated by using Design Expert. Fermentation is more valid at sterile condition with 52.56% difference with non-sterile condition. The optimum pH is 6.75 and optimum agitation is 176.56 rpm. The effect of pH was not significant with only 0.88% contribution and the interactions are more significant for agitation speed at 5.12% of contribution with 8.77% of error from expected result.

ABSTRAK

Setakat ini, bioetanol merupakan salah satu bahan api berasaskan benda hidup yang penting di dunia.Ini berikutan oleh proses teknologi yang telah dicapai dan terbukti dalam lingkungan bahan mentah yang sesuai bagi produksinya.Objektif kajian ini adalah untuk mengkaji kesan pH (3-7) dan kelajuan goncangan (110-250rpm) yang signifikan terhadap proses fermentasi getah perahan dari batang pokok kelapa sawit menggunakan yis *Saccharomyces cerevisiae* Kyokai no.7(ATCC 26422). bagi penghasilan bioetanol. Proses fermentasi yang sesuai telah direka bagi mendapatkan pengesahan fermentasi dan profil fermentasi seterusnya keputusan data untuk digunakan dalam kajian kesan parameter bagi keseluruhan proses pada beberapa titik masa yang telah dipilih. Data kepekatan etanol, glucosa dan jisim bio telah dikumpul dan dianalisa. ANOVA telah dijalankan bagi menentukan interaksi parameter dengan proses setelah kesan utama dianggarkan menggunakan perisian Design Expert. Fermentasi lebih sah pada medium steril dengan perbezaan 52.56% dengan medium yang tidak disterilkan. Nilai optimum pH dan kelajuan goncangan masing-masing adalah 6.75 dan 176.56 rpm. Kesan pH adalah tidak signifikan dengan hanya 0.88% sumbangan berbanding kesan kelajuan goncangan dengan 5.12% sumbangan dan ralat keseluruhan sebanyak 8.77% daripada nilai jangkaan.

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LIST OF ABBREVIATIONS

%	-	Percent/percentage
°C	-	degree Celcius
µm	-	micrometer
ANOVA	-	Analysis of Variance
DI	-	Distilled water
DNS	-	Di-Nitro Salicylic Acid
FID-GC	-	Flame ionization detector-gas chromatography
g	-	gram
g/L	-	gram per Liter
h	-	hour
HPLC	-	High Performance Chromatography
ID	-	Internal diameter
KJ	-	KiloJoule
L	-	Liter
m	-	meter
mg/L	-	milligram per Liter
min	-	minutes
mm	-	milimeter
N	-	Normality
no.	-	number
OD	-	Optical Density
OPT	-	Oil palm trunk
rpm	-	rotation or revolution per minutes
v/v	-	volume per volume
Y	-	Yield

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CHAPTER 1

INTRODUCTION

1.1 General

The dependence of the global economy on fossil-derived fuels, coupled with political instability in oil-producing countries, has pushed petroleum prices near all-time highs. Demand for energy is expected to increase more than 50% by 2025, mostly due to emerging economies-India and China. Increased use of fossil fuels will also increase atmospheric carbon dioxide, hastening the global warming crisis. Thus, there is an ongoing quest to develop sustainable, affordable, and environmentally sound energy from renewable and are an environmentally clean energy source, and have potential to significantly reduce consumption of fossil fuels (Soetaert and Vandamme, 2009). Bioethanol is one form of renewable energy source that is fast gaining foothold as potential fuel to power automotive engine. Contrary to gasoline which is refined through distilling crude fossil fuel, bioethanol can be synthesized from the starchy parts of natural plants or so called biomass. Microscopic yeast cells break down the starch and water, creating the so called bioethanol and carbon dioxide as end products. Bioethanol burns to produce carbon dioxide and water in complete combustion, a process akin to gasoline. It also possesses a high octane fuel, subsequently has replaced lead as an octane enhancer in petrol (Cheng *et al.*,2007). Alternatively, biomass and any wastes that result from its processing or consumption could be converted directly into synthetic organic fuel if suitable conversion process were available (Klass, 1998).

1.2 Problem Statement

Nowadays, energy for industrial, commercial and residential purposes, electricity generation and transportation is primarily supplied by fossil fuels and nuclear power. It is now believed that climate change is strongly linked to greenhouse gases in the atmosphere, and that the human activity especially through the combustion of fossils fuels is the major contributing factor (Smith, 2009). Moreover, thousands of tonnes of Oil Palm Trunk (OPT) that produced annually in Malaysia also has a significant effect on the environment, particularly due to the green house gas (GHG) that was released during the decomposition of OPT (Deris *et al.,* 2006).So, using the biomass to produce biofuels was far much more to environment concern to reduce of the greenhouse effect.

The production of raw biomass material and its subsequent conversion to fuels creates local jobs, provide regional economic development, and an increase farm and forestry incomes (Mulchandani, 2004). However, Columnist George Monbiot argues that using biomass to produce fuels would cause widespread hunger (Garza and Gale, 2007). However, in this case, we are using the waste from the palm oil industry, instead of feed sources. This will ensure constant supply of oil palm trunk that is regularly discharged from plantation sites without worried in lack of food sources (Yutaka *et al.,* 2009).

The environment benefits associated with the use of ethanol include a net reduction of carbon dioxide emissions and improved waste utilization. However, the cost of ethanol manufacture remains relatively high (Mulchandani, 2004).The batch processing for the production of bioethanol cost higher compare to continuous process. It is because we continuously withdraw the product without needing to run over the whole process in a batch processing plant.

1.3 Objective

The purpose of this experiment is to study the optimum condition for production of bioethanol from oil palm trunk (OPT) sap by yeast *Saccharomyces cerevisiae* Kyokai no.7.

1.4 Scope

The scope of the study is to design a batch fermentation process for bioethanol production. Firstly, we need to obtain fermentation validation and fermentation profile before measuring the final product of fermentation by comparing the value of glucose and ethanol concentrations of product to the standard calibration curve that will be obtain earlier. Furthermore, is the screening of the effect parameter; pH and agitation, and finally finding the suitable pH and agitation that give high yield of bioethanol.

1.5 Rational Significance

Bioethanol is by far the most important biofuel worldwide. This is mainly a result of the proven and established process technology in addition to the wide range of raw materials suitable for its production (Gurjahn and Elvers, 2008). Bioethanol assists countries in the development of strategies to become more independent of crude oil while enables them to achieve their targets for CO_2 emission reduction helps to create jobs in rural area (Gurjahn and Elvers, 2008). Today, Malaysia Palm Oil assumes an even larger role in addressing significant issues – the dependency of depleting fossil fuels and the growing threat of global warming. A biofuel programme that converts palm oil wastes to bioethanol, works towards offering the world a cleaner, economical friendly, renewable fuel (Govindaswamy and Lane, 2010).

CHAPTER 2

LITERATURE REVIEW

2.1 Oil Palm Trunk (OPT)

The oil palm tree (*Elaeis guineensis jacq.*) originates from West Africa where it grows in the wild and later was developed into an agricultural crop. It was introduced to Malaya, then Malaysia, by the British in early 1870's as an ornamental plant. In 1917, the first commercial planting took place in Tennamaran Estate in Selangor, laying the foundations for the vast oil palm plantations and the palm oil industry in Malaysia (Govindaswamy and Vane, 2010).

Malaysia currently accounts for 41 % of world palm oil production and 47% of world exports, and therefore also for 11 % and 25 % of the world's total production and exports of oils and fats. As the biggest producer and exporter of palm oil and palm oil products, Malaysia has an important role to play in fulfilling the growing global need for oils and fats in general (Hazira, 2006).

Oil palm is a monoecious crop as it bears both male and female flowers on the same tree (Hadyan, 2006). Oil palms are grown for their clusters of fruit, which can weigh 40-50 kg. Upon harvest, the drupe, pericarp and seeds are used for production of soap and edible vegetable oil (MPOCa, 2009). Each tree produces compact bunches weighing between 10 and 25 kilograms with 1000 to 3000 fruitlets per bunch (Hazira, 2006).

Oil palm tree normally passed their economic age, on an average after 25 years, and due to replanting. During replanting, the bole length of felled palm trunk is in the range of 7 m to 13 m, with a diameter of 45 cm to 65 cm, measured at breast height. The area due for replanting has to be multiplied with the average number of 134 palms ha^{-1}, or in volume of 1.638 m^3ha^{-1}. About 53.87 % (dry weight) of fiber bundles can be extracted from a trunk, with the remaining parts are the bark and parenchyma tissues which contribute to 14.45 % and 31.68 % of the dry weight of the trunk respectively (MPOCb, 2009). Table 2.1 show the proximate analysis of percent dry weigh of oil palm biomass by Yutaka *et al.*, (2009).

Table 2.1: Analysis of percent dry weigh of oil palm biomass

Oil palm biomass	Oil palm trunk	Oil palm fronds	Empty fruit bunch
Lignin	18.1	18.3	21.2
Hemicellulose	25.3	33.9	24
A-cellulose	45.9	46.6	41
Holocellulose	76.3	80.5	65.5
Ash	1.1	2.5	3.5
Alcohol benzene solubility	1.8	5	4.1

In spite of the huge production, the oil consists of only about 10 % of the total biomass produced in the plantation. The remainder consists of huge amount of oil palm wastes such as oil palm shells, mesocarp fibers and empty fruit bunch and oil palm fronds and oil palm trunk (from the field during replanting) (MPOCb, 2009), which contain variuos nutrient composition each as in Table 2.2.

Table 2.2: Nutrient composition of oil palm biomass

Oil palm biomass	Dry matters (tonne/hectare)	Nutrient (kg/hectare)			
		N	P	K	Mg
Trunks	75.5	368.2	35.5	527.4	88.3
Fronds(replanting)	14.4	150.1	13.9	193.9	24
Fronds(pruning)	10.4	5.4	10	139.4	17.2
Empty fruit bunches	1.6	107.9	0.4	35.3	2.7

According to Biocentrum-DTU (2010), the total amount of fermentable sugars in oil palm biomass is nevertheless higher than that expected value. The biomasses analysed were empty fruit bunches (EFB), trunks, fronds, fibres, palm kernel cake, EFB juice. All the biomasses were dried and grinded. Oil palm analysis by Biocentrum-DTU (2010), is as in Table 2.2 below.

Table 2.3: Oil palm biomass analysis

Biomass	EFB	Trunk	Fronds	Fibers	Kernel	EFB juice
Cellulose % of DM	39	59	42	21	6	7
Hemicelluloses % of DM	22	10	21	16	36	6
Lignin % of DM	29	11	23	43	36	10
Glucose g / g DM	0.43	0.65	0.47	0.23	0.07	0.07
Xylose g /g DM	0.26	0.12	0.24	0.18	0.4	0.07
Total fermentable g /g DM	0.69	0.77	0.71	0.41	0.47	0.14

Basically, the oil palm biomass contains about 18 – 21 % of lignin, and 65-80 % of holocellulose (a-cellulose and hemicellulose), which are more or less comparable with that of other wood or lignocellulosic materials (refer to Table 2.2). This makes the oil palm biomass is also suitable as a raw material for the production of bioethanol (MPOCb, 2009). Other than oil palm trunk, the other part oil palm biomass that could be use in producing bioethanol is Empty Fruit Bunch (EFB) and funds. However, according to Table 2.3 by Biocentrum-DTU (2010), oil palm trunk gives the highest yield of ethanol compare to the other. Kosugi *et al,* (2010) reported that oil palm trunk sap contained 13.1, 52.5 and 85.2 g/L glucose in the outer, middle and inner trunks, respectively.

Table 2.4: Ethanol yield from oil palm biomass

Biomass	EFB	Trunks	Funds
Glucose g/g/ DM	0.43	0.65	0.47
Xylose g/g DM	0.26	0.12	0.24
Ethanol L / ton DM	388	451	377

2.2 Rationale of the Current Research and Development in Malaysia

2.2.1 Production Of Bioethanol from Oil Palm Empty Fruit Bunch (EFB)

Conversion of bioethanol from EFB offers a simple yet effective treatment of waste from palm industry. By using EFB, the competition with food-based raw materials can be alleviated, thus preserving the price of food commodity. The abundance sources of oil palm-based biomass provide an impetus for sustainable generation of bioethanol as oil palm production constitutes major agricultural industry in Malaysia (Hadyan, 2006).

2.2.2 Production of Bioethanol from Palm Oil Mill Effluent (POME)

The process to extract oil from the EFB requires voluminous amount of water, mainly for sterilizing the fruits and for oil clarification, resulting in the discharge of about 2.5 m^3 of effluent per tonne of crude oil processed. Fresh palm oil mill effluent, or POME characterised by high contents of organics and solids and it has been estimated that POME contributes to about 30 % of the total biochemical oxygen demand (BOD) load exerted on the Malaysian aquatic environment. So, POME was used to break down into sources of bioethanol rather than being one of the major sources of pollution in Malaysia (Hazira, 2006).

CHAPTER 3

METHODOLOGY

3.1 Medium and Reagent Preparation

3.1.1 Medium Preparation

Four types of medium are used throughout this research which is nutrient agar (NA), nutrient broth (NB) and also palm oil trunks sap.

Nutrient agar plate (NA) is prepared by mixing 20 g of agar, 10 g of peptone and 5 g of yeast extract with 900 mL of distilled water in 1 Liter of Schott Bottle. 5 g of glucose was mixed with 100 mL of distilled water in 250 mL flask. Both flask are then covered with aluminum foil and being autoclaved for 20 minutes at 121 °C. Glucose is added in NA after autoclave to avoid Maillard reaction (brownization). After temperature has dropped under 90 °C, agar is pour into sterilized Petri plate. The plates are leaved undisturbed until the agar solidifies. All plates are closed and seal before kept in refrigerator at 4 °C.

Nutrient broth (NB) is prepared by mixing the same portion of nutrient ingredients (yeast extract, peptone and distilled water) as in NA preparation accept there are no agar powder. Glucose is prepared and is added in NB after autoclave. The solution is then mix well before refrigerate at 4 °C.

The palm oil trunks were freshly obtained from Jerantut Plantation, Pahang, Malaysia. Palm oil trees age more than 15 years is chosen. The sap was collected (refer to Figure 3.1) by pressing middle of trunks with press machine within less than 24 hour after trees was cut down. The sap was immediately kept at -20°C to avoid any/further microbial reaction before use.

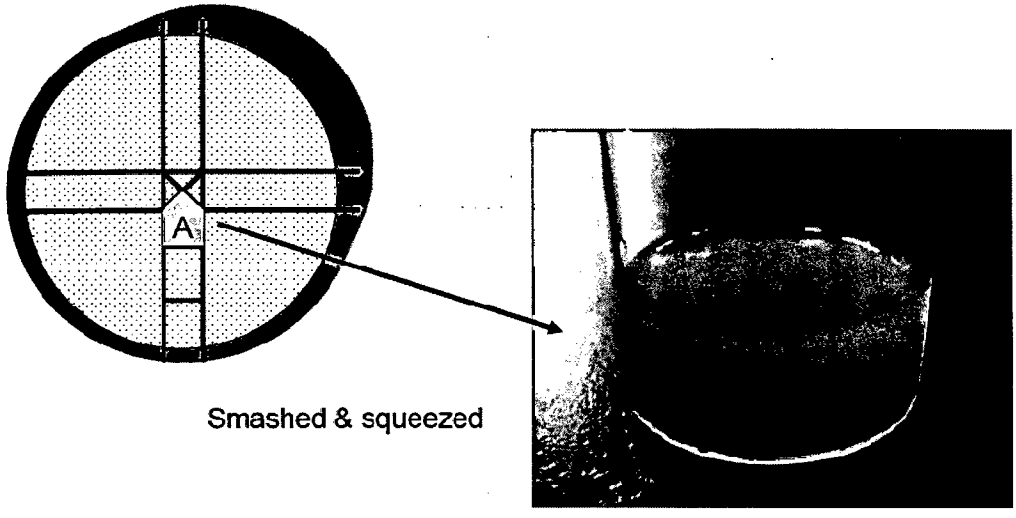

Figure 3.1: Oil palm trunk sap

3.1.2 Reagent Preparation

The reagent use include DNS Reagent, 10 g of NaOH , 182 g of Sodium Potassium Tartarate, 2 g of Phenol, 0.5 g of Sodium Sulphite, and 10 g Dinitrosalicylic acid. The entire ingredient is dissolved in 600 mL distilled water before top up to 1 Liter in amber bottle or cover with aluminum foil. Then it is stir overnight. It is keep at 4 °C in refrigerator.

3.2 Microorganism and medium

3.2.1 Pure Culture Preparation

Strain is prepared by dissolving yeast into NB and incubates for 1 day (100 rpm, 30 °C) to form yeast suspension. The type of yeast use is *Saccharomyces cerevisiae* Kyokai no. 7 (ATCC 26422) which has been obtained from American Type Culture Collection. Next, yeast suspensions are streaked on new agar plate and incubate 2 - 3 days at 30 °C. The strain was kept at 4 °C and sub-cultured every month on new NA to maintain the pure culture supply continuity.

3.2.2 Inoculum Preparation

About 3 - 4 loop of pure culture is transferred from agar plate into 100 mL of sterile NB in shake flask. Strain was incubated for 12 - 18 hours at 30 °C and 150 rpm until reached standard initial concentration. The cell concentration was standardized to 0.2 - 0.4 g/L (OD = 4.2) determined by turbidimetry at 600 nm. All procedures are done aseptically and experiment is run duplicate for all parts.

3.3 Fermentation

3.3.1 Preparation of fermentation validation

Fermentation validation is done to validate sterile and non-sterile condition OPT sap. Fermentations were carried out in a 500 mL Erlenmeyer flask with 250 mL total working volume. The sterile sap was filtered with 9.0 μm filter and autoclaved at 121 °C for 15 minutes before use. Fermentation samples were collected and analyzed at 0, 24, 36 and 48 h.

3.3.2 Preparation of fermentation profile

Fermentation profile is done to estimate initial residence time to start batch fermentation after placing yeast suspension. 10% (v/v) of inoculum suspension from activated yeast flask is transferred into sterilized palm oil trunks sap. All fermentation condition is fixed to, temperature; 30 °C, pH; control/unadjusted; inoculums size; 10 % v/v and agitation speed with 180 rpm.

3.3.3 Preparation of fermentation optimization of parameter

Batch fermentation process has been design for the optimization of bioethanol production. All data have been set as Table 3.1 below. Each set have been duplicated in the first run and repeated in the next run to obtain more accurate result.

Table 3.1: Fermentation condition for the fermentation parameter optimization

Flask no.	Temperature (°C)	pH	Agitation	Inoculum size (%)
1	40	3	250	5
2	40	7	250	5
3	25	3	250	15
4	40	7	110	5
5	25	7	250	15
6	40	7	250	15
7	25	3	110	15
8	40	3	110	5
9	40	3	110	15
10	40	3	250	15
11	40	7	110	15
12	25	7	250	5
13	25	7	110	15
14	25	7	110	5
15	25	3	110	5
16	25	3	250	5

Oil palm trunk (OPT) sap is melted after carried out from -20 °C freezer. After it completely turn into liquid, shake well and filter by coffee filter. The pH of the melted sap that has been filtered by coffee filter needed to be checked. Next, the pH was adjusted with either using Natrium Hyroxide (NaOH) 2N for alkali condition or using 6 % Sulphuric Acid (H_2SO_4) for acidic condition of sap.

After that, 225 mL of sap is measured for each flask using measuring cylinder. The flask is then closed with stopper and was wrapped with aluminum foil after put syringe filter. Then, the flask was autoclaved using Hirayama Autoclave for Mode 2. After inoculums have been transferred, the rpm of Incubator Shaker is then adjusted with the rpm different according to Table 3.1 and fermentation started.

3.4 Method of Analysis

3.4.1 Cell dry weight (CDW)

1.5 mL of sample is centrifuge at 10,000 rpm for 5 minutes. The supernatant is removed for glucose and ethanol analysis. The pellet is then rinsed two times with distilled water. After that, 1.5 mL of distilled water is added into tube containing rinsed pellet and is well mixed by using vortex. Immediately, 0.5 mL of sample is taken for Optical Density (OD) analysis. The absorbance for each sample during fermentation was measured at 660 nm using UV-Vis spectrophotometer, U-1800. The instrument was zeroed using uninoculated sterile medium as blank. Absorbance values were converted cell dry weight concentration by using standard curve obtained from absorbance versus cell dry weight calibration $y = 12.907x - 2.185$, where Y is biomass (g-dry biomass/L) and X is absorbance at 600 nm. For cell dry weight determination, pellets were dried at 60°C until constant weight after rinsed twice with distilled water.

3.4.2 Glucose Concentration

Glucose assay is used to determine glucose concentration during fermentation (Miller, 1959). Ratio 1: 2: 10 (sample: DNS reagent: distilled water) is used for sample and glucose standard. 0.2 mL of DNS reagent is added to 0.1 mL of sample in test tube before it is capped with parafilm.

Next, test tube is boiled in 90 °C water for 5 minutes to develop red-brown color. After it is cooled, 1 mL of distilled water is added and mixture is shaken vigorously. Finally, OD is read by using UV-Visible Spectrophotometer, at 540 nm wavelength. Proportion for glucose standard is prepared and mixed as in Table 3.1 below.

Table 3.2: Proportion for glucose standard

	Volume in Test Tube (mL)					
	Blank	1	2	3	4	5
Stock solution	0.0	0.1	0.2	0.3	0.4	0.5
Distilled water	1.0	0.9	0.8	0.7	0.6	0.5
DNS reagent	1.0	1.0	1.0	1.0	1.0	1.0

3.4.3 Ethanol Concentration

Ethanol concentrations were determined by a gas chromatograph (HP Agilent) equipped with a flame ionization detector (FID) and Innowax column (30 m x 12 mm ID x 2 μm film thickness). Temperature of injector and detector were 150 °C and 200 °C, respectively. The carrier gas was nitrogen at a flow rate of 15 m. The whole fermentation process for bioethanol production can be summarized in Figure 3.1.

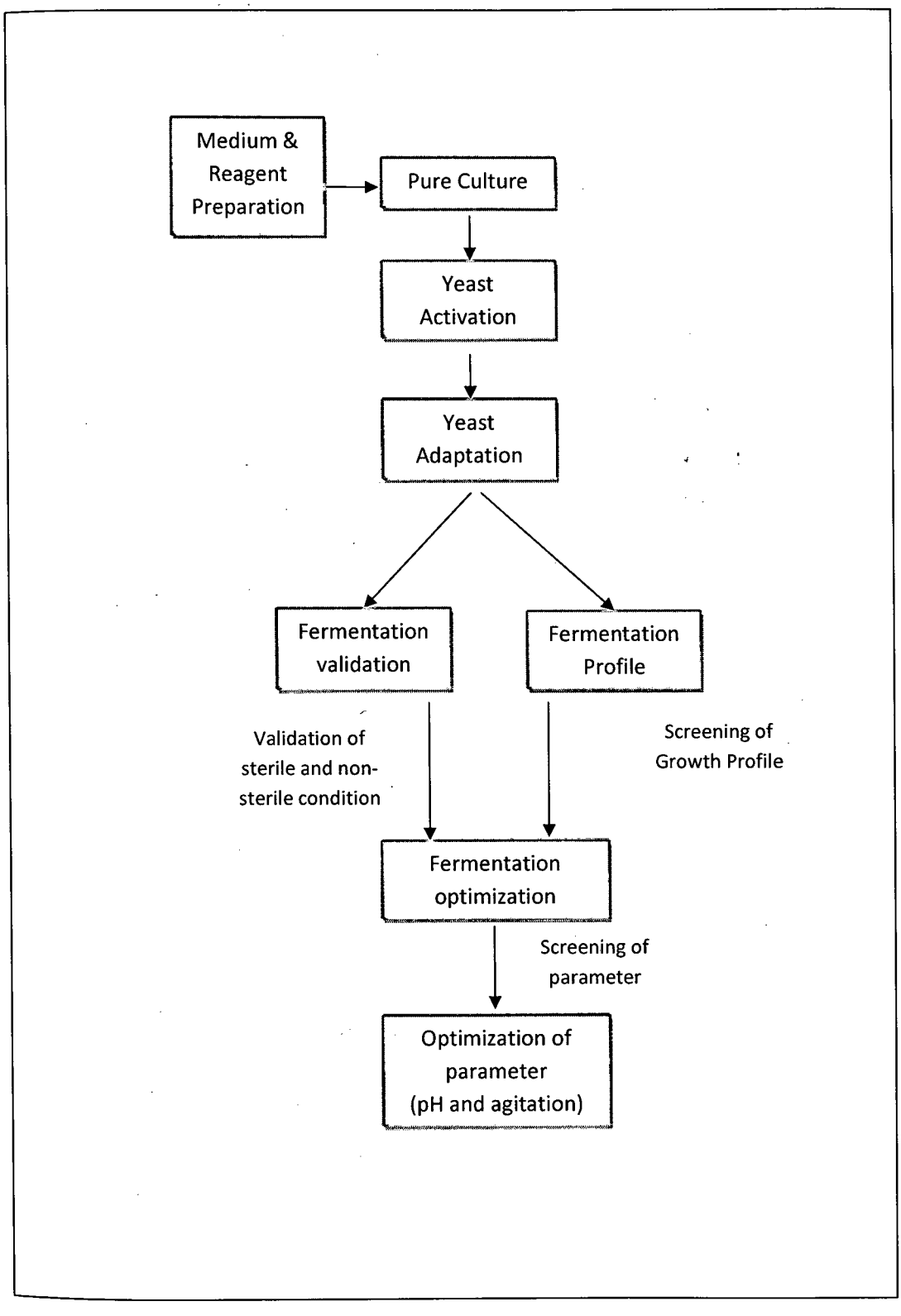

Figure 3.1: The diagram for the whole fermentation process for bioethanol production

CHAPTER 4

RESULT AND DISCUSSION

4.1 Fermentation validation

During study, we expected that the productivity of ethanol will increase with the decreasing of glucose level from the biomass that used and from the Figure 4.1, the expectation well fulfill. The reaction is exothermic and can be summarized as following equation:

$$C_6H_{12}O_6 + 2\ P + 2\ ADP \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP \quad \Delta H = -156Kj$$

The conversion of 1kg glucose leads to the formation of 511g bioethanol, 489g CO_2 and 867 kJ of heat (MPOCa, 2009).

Bio-ethanol fermentation performance can be evaluated on the basis of ethanol conversion from initial sugar in the sap.

Y p/s = (final concentration of bio-ethanol/ initial concentration of sugar)/0.511

In order to obtain fermentation data to be used in optimization of parameter; agitation and pH, we first need to obtain fermentation validation. Fermentation validation is done to validate sterile and non-sterile condition of oil palm trunk sap. All fermentation condition is fixed to, temperature; 30 °C, pH; control/unadjusted; inoculums size; 10 % v/v and; agitation speed with 180 rpm.

Fermentation samples were collected and analyzed at 0, 24, 36 and 48 h. Graph were plotted as in Figure 4.1.